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Essential fatty acids deficient diet modulates N-Acylethanolamide profile in rat's tissues

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ABSTRACT

No data are available on whether a diet deficient of the essential fatty acids is able to modulate tissue levels of endocannabinoids and congeners.

Male rats fed for 12 weeks a diet deficient of essential fatty acids, palmitic and oleic acids (EFAD), replaced with saturated fatty acids (SAFA), showed lowered n-3 and n-6 PUFAs levels in plasma, liver and adipose tissue, with concomitant steep increase of oleic and mead acids, while in hypothalamus no changes in PUFA concentration were detected and only palmitoleic acid was found increased. We found a reduction of anandamide and palmitoylethanolamide in liver and brain, while oleoylethanolamide increased significantly in liver and adipose tissue, associated to a 50 % body weight decrease.

Changes in N-acylethanolamide profile may contribute to body weight reduction distinctive of EFA deficiency.

1. Introduction

Several lines of evidence show that dietary fatty acids may influence endocannabinoids (ECs) and their congeners tissue profiles *in vitro* [1] and *in vivo* [2]. Most of the studies aimed at evaluating whether changes in arachidonic acid (ARA, 20:4n-6) tissue phospholipids (PL) content were able to modulate the main ECs anandamide (AEA) and 2-arachidonoylglycerol (2-AG) [3]. Two major strategies have been described in the literature, reduction of ARA by increasing dietary n-3 long chain polyunsaturated fatty acids (n-3 LCPUFAs), i.e. eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) [4], or increasing it by feeding ARA precursor, linoleic acid (LA, 18:2n-6) [5]. In humans, it has been shown that increasing dietary EPA and DHA was able to decrease 2-AG [6] or AEA [7]; however, other nutritional strategies to increase tissue n-3 LCPUFAs, as demonstrated with intake of cheese enriched in alpha-linolenic (ALA, 18:3n-3), conjugated linoleic (CLA, 18:2c9t11) and vaccenic (VA, 18:1t11) acids, were able to significantly decrease plasma levels of AEA in hypercholesterolemic patients [8]. On the other hand, no data are available on whether a diet deficient in n-3 and in n-6 fatty acids replaced by saturated fatty acids (SAFA) is able to modify EC and congeners tissue profile. It is well known that intake of an essential fatty acid deficient (EFAD) diet is characterized by growth retardation and weight loss [9], even though the mechanism of action is not quite well understood.

ARA possesses essential functions, particularly in cellular signalling via its role of precursors for numerous derivatives such as prostaglandins, leukotrienes, hepoxilins and other eicosanoids including ECs which strongly influence body composition homeostasis [10]. Symptoms of n-6 fatty acid deficiency involves, initially, scaly skin, decreased growth and increased transepidermal water loss, all symptoms that seem to be attributable to an essential structural role of LA in O-acylated ceramides of the epidermal water permeability barrier [11]. A dietary intake of around 1 en% of LA should be enough to prevent these symptoms in animals and in humans [12].

An early symptom of n-3 fatty acids deficiency is delayed brain development due to their essential role for proper brain function, probably via their incorporation into specific cellular PL [13]. Humans in the Western world ingest far less n-3 fatty acids (around 1–3 g/d, mainly ALA and, to a lesser extent, EPA and DHA) than n-6 fatty acids (10–20 g/d, mainly LA). The dietary intake of EPA and DHA varies greatly between individuals and between geographical populations and is mainly related to the dietary intake of seafood. It is well evident that EPA and DHA can inhibit the *in vitro* production of ARA-derived eicosanoids [14], but the *in vivo* formation seems to be much less influenced by dietary intake of EPA and DHA [14]. Generally, a dietary intake of several grams per day of EPA and DHA for many weeks is necessary for

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observing a moderate decrease in the *in vivo* production of ARA-derived eicosanoids [15].

Calculation of the endogenous production in humans of ARA from LA indicates that this may be a bit higher than the dietary intake, i.e. 180–800 mg/day, depending on the dietary intake of LCPUFAs [16].

Generally, the levels of ARA in tissue PL are to some degree influenced by the dietary intake of different polyunsaturated fatty acids (PUFA) but the brain seems to be less influenced, especially the non-growing adult brain [17].

The impairment of AEA biosynthesis, due to a lower precursor availability, may influence the biosynthesis of other N- congeners such as oleoylethanolamide (OEA) and palmitoylethanolamide (PEA), which are strong PPARalpha ligands and have been shown to possess opposite physiological activities with respect to AEA, particularly in terms of lipid and energy metabolism [18,19]. Therefore, in this study we aimed at evaluating whether changes in tissue fatty acid profile, induced by a EFAD diet, by affecting both n-6 and n-3 fatty acids tissue concentrations, are able to influence tissue levels of the ECs, AEA and 2-AG, and the congeners PEA and OEA.

2. Materials and methods

2.1. Experimental diets

The EFAD diet (Lab. Piccioni, Segrate, Italy) was composed as follows (for 100 g of food, dry weight): casein vitamin-free, 18%; sucrose plus maize starch, 68.4%; cellulose, 2%; hydrogenated coconut oil, 4.6%; Hegsted salt, 4.8%; yeast, 2%; plus a vitamin integration, 0.2%. Fatty acid composition of the control and EFAD diets is depicted in Table 1.

2.2. Animals

Male Sprague–Dawley rats (Harlan, Italy) weighing 85–100 g were used. Animals were housed at a constant temperature of 22 ± 2 °C and 60% relative humidity, on a 12-h light/dark cycle (lights on at 7:00 a.m.), food and water available ad libitum. All experimental protocols were applied in strict accordance with international guidelines regulating the use of animals for scientific purposes.

One group of 20 rats received the EFAD diet, a second group (20 rats) was fed with an isocaloric standard laboratory chow (controls) for 12 weeks. The weight of the animals was monitored during the experimental period.

Table 1
Dietary fatty acid composition.

Fatty acids	mol % CTRL	EFAD
18:3n-3	5.20	0.03
18:2n-6	50.48	1.19
16:1n-9	1.06	0.09
18:1n-9	22.58	1.34
8:0	0.03	3.58
10:0	0.07	6.76
12:0	0.56	52.19
14:0	0.94	16.38
16:0	17.27	9.71
18:0	1.83	8.37
n-6/n-3 ratio	9.71	42.63
SAFA	20.69	96.99
MUFA	23.63	1.44
PUFA	55.67	1.22
PUFA > 20 C	ND	ND

Dietary fatty acid concentrations are expressed as mol% of total fatty acids. CTRL, control diet; EFAD, essential fatty acid deficient diet. Not detected (ND).

2.3. Measurement of fatty acids, endocannabinoids and related compounds

Total lipids from plasma, liver, adipose tissue and hypothalamus were extracted by the method of Folch et al. [20]. Total lipid quantification was performed by the method of Chiang et al. [21]. Aliquots of the lipid fraction were mildly saponified using a procedure in order to obtain free fatty acids (FFA) for HPLC analysis [22]. All reagents were HPLC grade and purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Separation of fatty acids was carried out with an Agilent 1100 HPLC system (Agilent, Palo Alto, CA, USA) equipped with a diode array detector. A C-18 Inertsil 5 ODS-2 Chrompack column (Chrompack International BV, Middleburg, The Netherlands), 5 μm particle size, 150 × 4.6 mm, was used with a mobile phase of $CH_3CN/H_2O/CH_3COOH$ (70/30/0.12, v/v/v) at a flow rate of 1.5 ml/min [23]. SAFA were measured as fatty acid methyl esters (FAMEs), by a gas chromatograph (Agilent, Model 6890, Palo Alto, CA, USA) equipped with split ratio of 20:1 injection port, a flame ionization detector (FID), an autosampler (Agilent, Model 7673, Palo Alto, CA, USA), a 100 m HP-88 fused capillary column (Agilent, Palo Alto, CA, USA). Data were analyzed by the Agilent ChemStation software system. Quantification of endocannabinoids AEA and 2-AG, and other N-acylethanolamide (NAE) compounds OEA and PEA, was carried out by liquid-chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS), and using selected ion monitoring (SIM) at M+1 values for the compounds and their deuterated homologues. Internal deuterated standards for AEA, 2-AG, OEA and PEA quantification by isotope dilution ($[^2H]_8$AEA, $[^2H]_5$2AG, $[^2H]_2$ OEA, $[^2H]_4$ PEA) were purchased from Cayman Chemicals (MI, USA). A C-18 Zorbax Eclipse Plus column (Agilent, Palo Alto, CA, USA) 5 μm particle size, 50 × 4.6 mm, was used with a mobile phase of $CH_3OH/H_2O/CH_3COOH$ (80/20/0.3, v/v/v) at a flow rate of 0.5 ml/min. A typical chromatogram of NAEs is reported in Fig. 1. Endocannabinoids, PEA and OEA were then expressed as mol% of the sum of total fatty acids measured in each corresponding tissue.

2.4. Statistical analysis

Quantitative data are presented as means ± SEM and were analyzed by t-student. We assumed p-value <0.05 to establish statistical significance.

3. Results

Feeding EFAD resulted in an almost 50% reduction of body weight (Fig. 2A) and of 20% of BMI (Fig. 2B), suggesting a growth retardation. In fact, the length of the rats in cm (control 24.50 ± 0.87 vs EFAD 20.57 ± 0.93) was significantly different ($p < 0.05$).

As shown in Table 2, feeding EFAD dramatically impacts on plasma fatty acid profile with a strong reduction of n-3 LCPUFAs of about 80% while ARA decreased by 40%. n-3 and n-6 PUFAs were mainly replaced by mead acid and its precursor oleic acid (OA, 18:1n9) and the other MUFA palmitoleic acid (POA, 16:1). Despite the high dietary SAFA concentration in EFAD, total tissue SAFA did not change significantly.

Changes of liver (Table 3) and adipose tissue (Table 4) fatty acid profiles reflected those in plasma. Interestingly, we did not find any change in fatty acid profile of the hypothalamus, besides an almost 3-fold increase of POA (Table 5).

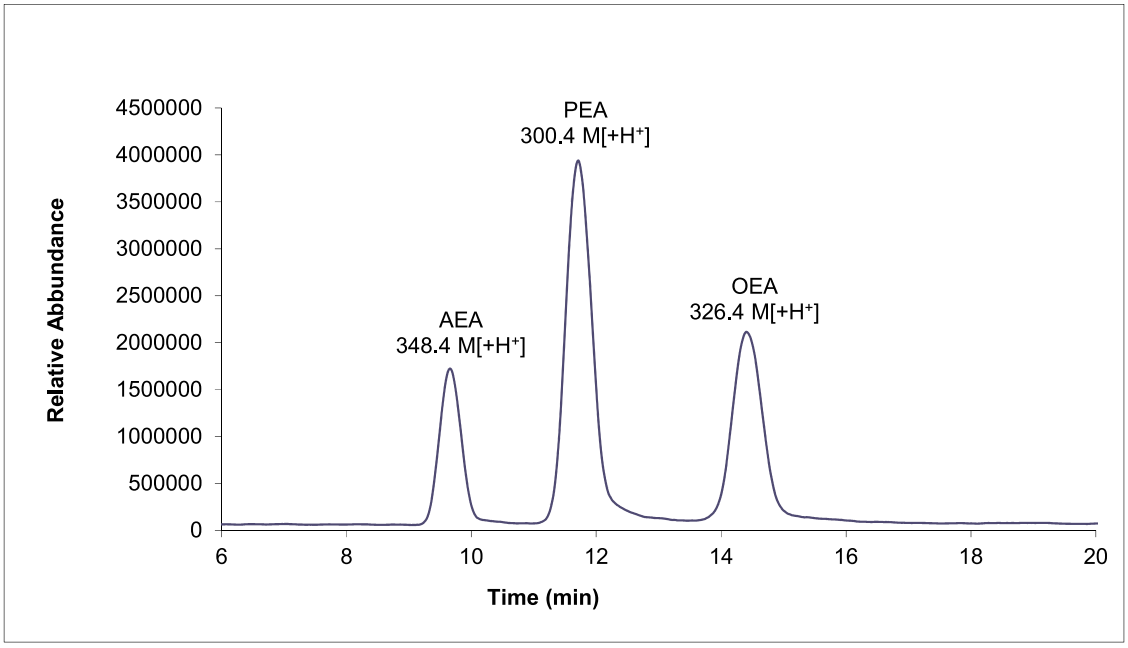

Fig. 1. Typical LC–MS separation of anandamide (AEA), palmitoylethanolamide (PEA) and oleoylethanolamide (OEA). Chromatographic conditions are described in materials and methods.

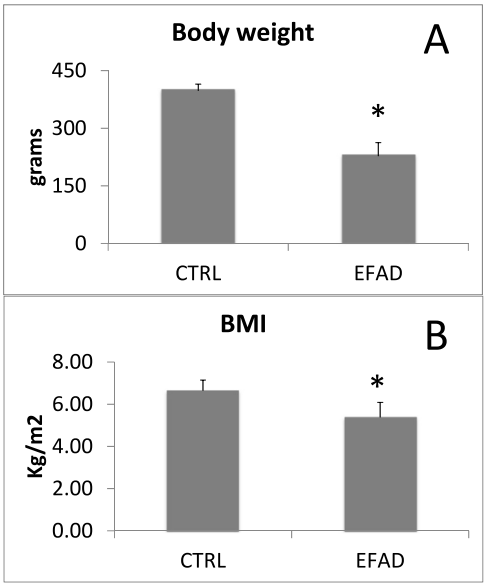

fig. 2. Body weight (panel A) and BMI (panel B) of rats fed control diet (CTRL) or essential fatty acid deficient diet (EFAD). * Significantly different from control ($p < 0.05$).

Endocannabinoids, PEA and OEA were analyzed from liver, AT and hypothalamus, however only significant changes are reported. AEA was found decreased in liver (Fig. 3A) and hypothalamus (Fig. 4A), while 2-AG didn't change significantly (data not shown). OEA increased significantly in the liver (Fig. 3B) and adipose tissue (Fig. 5) and did not change in the hypothalamus (data not shown). On the other hand, PEA strongly decreased in liver (Fig. 3C) and hypothalamus (Fig. 4B), apparently without any correlation with the content of its precursor palmitic acid (PA, 16:0).

Table 2
Plasma fatty acid profile

	Plasma (mol%) CTRL			EFAD			
18:3n-3	0.58	±	0.05	0.09	±	0.01	*
18:4n-3	0.04	±	0.01	0.04	±	0.00	
20:5n-3	0.63	±	0.04	0.11	±	0.02	*
22:5n-3	0.50	±	0.01	0.00	±	0.00	*
22:6n-3	2.50	±	0.23	0.85	±	0.08	*
18:2n-6	18.95	±	1.22	3.31	±	0.46	*
18:3n-6	0.18	±	0.02	0.19	±	0.02	
20:3n-6	0.22	±	0.03	0.38	±	0.04	*
20:4n-6	16.46	±	1.49	9.09	±	0.68	*
22:4n-6	0.15	±	0.01	0.09	±	0.03	*
22:5n-6	0.06	±	0.01	0.74	±	0.05	*
14:1n-5	0.09	±	0.01	0.05	±	0.05	
16:1n-7	2.94	±	0.65	5.29	±	0.17	*
18:1n-9	12.92	±	0.70	26.59	±	0.57	*
20:3n-9	0.08	±	0.03	5.49	±	0.39	*
12:0	0.31	±	0.12	1.09	±	0.03	*
14:0	1.26	±	0.27	2.33	±	0.08	*
16:0	32.18	±	1.33	31.23	±	0.39	
18:0	8.82	±	0.16	11.11	±	0.22	*
SAFA	43.75	±	1.53	45.11	±	1.73	
MUFA	15.95	±	1.36	31.92	±	0.43	*
PUFAn-6	35.68	±	2.77	13.25	±	1.49	*
PUFAn-3	4.38	±	0.30	1.08	±	0.15	*

Plasma fatty acid concentrations (mean ± SEM), $n = 20$/group, are expressed as mol% of total fatty acids.

* Significantly different from control ($p < 0.05$). CTRL, rats fed control diet; EFAD, rats fed essential fatty acid deficient diet.

4. Discussion

Our data confirm that reduced intake of essential fatty acids strongly induced PUFA biosynthesis by maximizing fatty acid elongation and desaturation as shown by the increase in delta 9 desaturase products, OA and POA, and those from delta 5 and delta 6 desaturases

Table 3
Liver fatty acid profile.

	Liver (mol%) CTRL			EFAD			
18:3n-3	0.41	±	0.03	0.06	±	0.01	*
18:4n-3	0.03	±	0.01	0.00	±	0.00	*
20:5n-3	0.72	±	0.06	0.24	±	0.01	*
22:5n-3	0.79	±	0.04	0.00	±	0.00	*
22:6n-3	6.33	±	0.36	2.49	±	0.34	*
18:2n-6	21.97	±	1.39	5.58	±	1.40	*
18:3n-6	0.19	±	0.03	0.20	±	0.02	
20:3n-6	0.40	±	0.02	0.52	±	0.09	*
20:4n-6	18.83	±	0.69	13.04	±	0.62	*
22:4n-6	0.23	±	0.02	0.17	±	0.01	*
22:5n-6	0.14	±	0.07	1.61	±	0.14	*
16:1n-7	1.85	±	0.33	4.85	±	0.53	*
18:1n-9	11.88	±	0.95	25.91	±	0.94	*
20:3n-9	0.37	±	0.38	7.49	±	0.28	*
12:0	0.03	±	0.00	0.10	±	0.00	*
14:0	0.28	±	0.03	0.54	±	0.02	*
16:0	22.06	±	0.53	21.31	±	0.23	*
18:0	12.65	±	0.40	14.45	±	0.40	*
SAFA	35.44	±	0.76	36.02	±	0.97	
MUFA	13.73	±	1.25	30.77	±	1.46	*
PUFA	50.64	±	2.02	31.81	±	1.90	*

Liver fatty acid concentrations (mean ± SEM), n = 20/group, are expressed as mol% of total fatty acids.

* Significantly different from control ($p < 0.05$). CTRL, rats fed control diet; EFAD, rats fed essential fatty acid deficient diet.

Table 4
Adipose tissue fatty acid profile.

	Adipose tissue (mol%) CTRL			EFAD			
18:3n-3	1.04	±	0.17	0.02	±	0.00	*
18:4n-3	0.03	±	0.01	0.00	±	0.00	*
20:5n-3	0.03	±	0.01	0.00	±	0.00	*
22:5n-3	0.04	±	0.01	0.00	±	0.00	*
22:6n-3	0.07	±	0.03	0.01	±	0.00	*
18:2n-6	16.91	±	2.36	0.47	±	0.02	*
18:3n-6	0.06	±	0.01	0.01	±	0.00	*
20:3n-6	0.07	±	0.03	0.02	±	0.01	*
20:4n-6	0.18	±	0.05	0.10	±	0.00	*
22:4n-6	0.03	±	0.01	0.00	±	0.00	*
14:1n-5	0.17	±	0.03	0.37	±	0.02	*
16:1n-7	7.82	±	1.07	7.74	±	0.53	
18:1n-9	27.69	±	1.19	39.31	±	0.20	*
20:3n-9	0.01	±	0.00	0.05	±	0.01	*
12:0	0.27	±	0.05	4.85	±	0.19	*
14:0	2.63	±	0.08	5.56	±	0.14	*
16:0	38.75	±	0.65	35.38	±	0.45	*
18:0	3.51	±	0.20	5.61	±	0.07	*
SAFA	45.62	±	0.64	51.55	±	0.75	*
MUFA	35.675	±	2.13	47.41	±	0.75	*
PUFA	18.62	±	2.67	0.69	±	0.04	*

Adipose tissue fatty acid concentrations (mean ± SEM), $n = 20$/group, are expressed as mol% of total fatty acids.

* Significantly different from control ($p < 0.05$). CTRL, rats fed control diet; EFAD, rats fed essential fatty acid deficient diet.

20:3n-9.

The dramatic changes in tissue fatty acid profile resulted in marked alterations of tissue NAEs in liver and adipose tissues, but not in the hypothalamus. In fact, in liver and adipose tissue the higher levels of OA corresponded to an increase of OEA, while only in the liver a decrease of ARA was associated to a reduction of AEA but not of 2-AG.

Interestingly, we did not find any sign of EFAD in the hypothalamus.

Table 5
Hypothalamus fatty acid profile.

	Hypothalamus (mol%) CTRL			EFAD			
18:3n-3	0.08	±	0.02	0.10	±	0.03	
20:5n-3	0.04	±	0.01	0.04	±	0.00	
22:5n-3	0.24	±	0.06	0.18	±	0.00	
22:6n-3	11.32	±	0.56	11.28	±	0.60	
18:2n-6	0.94	±	0.08	0.87	±	0.15	
20:3n-6	0.18	±	0.06	0.15	±	0.03	
20:4n-6	10.57	±	1.78	10.02	±	0.60	
22:4n-6	2.55	±	0.08	2.68	±	0.18	
16:1n-7	0.64	±	0.06	1.72	±	0.18	*
18:1n-9	24.59	±	1.01	25.12	±	1.35	
20:3n-9	0.66	±	0.10	0.53	±	0.08	
12:0	0.37	±	0.24	0.57	±	0.39	
14:0	0.66	±	0.30	0.93	±	0.05	
16:0	26.16	±	0.83	25.40	±	1.49	
18:0	18.45	±	0.76	17.33	±	1.27	
SAFA	47.92	±	1.78	46.16	±	2.78	
MUFA	25.22	±	1.05	26.84	±	1.20	
PUFA	26.85	±	2.17	27.00	±	1.58	

Hypothalamus fatty acid concentrations (mean ± SEM), $n = 20$/group, are expressed as mol% of total fatty acids.

* Significantly different from control ($p < 0.05$). CTRL, rats fed control diet; EFAD, rats fed essential fatty acid deficient diet.

We may speculate that the relatively high content of medium chain fatty acids in the diet may favor their peroxisomal beta-oxidation in the brain, sparing PUFAs from oxidation and, on the other hand, producing acetate unit for de novo lipogenesis (DNL) and PUFA biosynthesis from their precursors ALA and LA even though they are present in the diet at very low concentration. This hypothesis may also explain the 200% increase of POA as both, product of DNL or peroxisomal beta oxidation of OA. In fact, it is unlikely that it comes from blood since other fatty acids found increased in plasma, such as 20:3n-9, were not found increased in hypothalamus. Furthermore, it has been shown that delta 9 desaturase activity is enhanced in conditions of neurodegenerative diseases [24]. In our study apparently, these mechanisms may contribute to maintain optimal levels of ARA and DHA but also PA in hypothalamus. However, we found in the same brain area a steep decrease of AEA and PEA, which seems to suggest that their biosynthesis may be impaired in an attempt to spare their precursors to maintain membrane homeostasis.

Few studies focused on changes in brain of ECs and congeners by dietary means. One study found that feeding mice with extremely high levels of fish oil, resulted in decreased levels of 2-AG in the brain [25]. Hanus et al. [26] observed that a fat-rich diet (46 energy%, mainly soybean oil) for 12 days induced a 60% fall in mouse brain levels of 2-AG.

Feeding adult rats for 1 week with diets comprising 36 energy% from different fats SAFA, PUFA and MUFA, significantly changed brain EC levels [27]. A high-fat diet (60 energy% for 14 weeks) has been reported to increase levels of AEA in mouse liver [28] and, interestingly, the authors suggested that this could contribute to diet-induced obesity via activation of CB1 receptors in the liver.

Another study has shown that feeding suckling piglets with a milk formula deficient in ARA decreased the brain levels of AEA and 2-AG and that the levels could be increased by adding ARA plus DHA to the milk formula, and by supplementing mice with 0.5 weight% of ARA for 58 days had dramatically increased brain levels of AEA [29].

We have previously shown that krill oil in relative low concentration in the diet was able to decrease 2-AG in brain in obese Zucker rats [3]. In these studies, changes in EC levels were found associated with

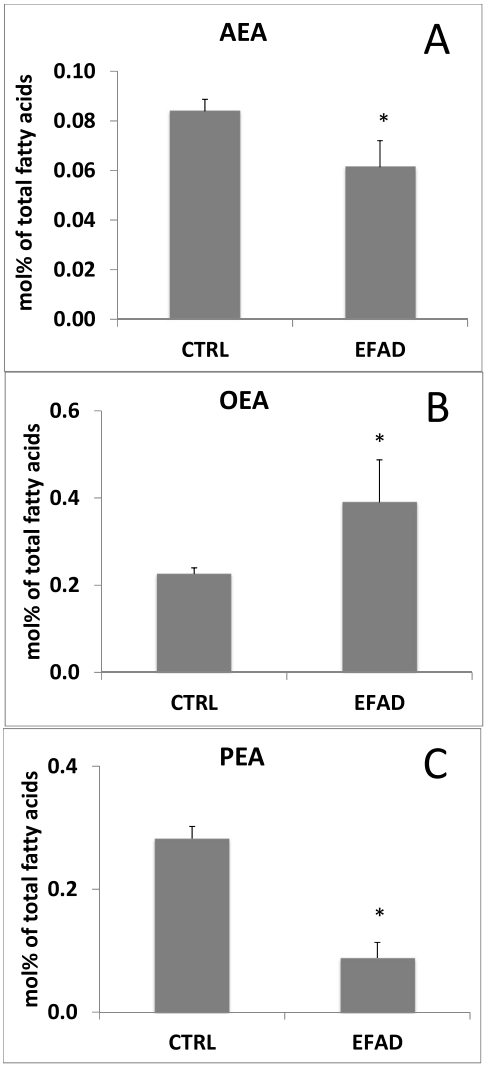

Fig. 3. Anandamide (AEA) (panel A), oleoylethanolamide (OEA) (panel B) and palmitoylethanolamide (PEA) (panel C) levels in the liver of rats fed control diet (CTRL) or essential fatty acid deficient diet (EFAD), values are expressed as mol % of total fatty acids. * Significantly different from control ($p < 0.05$).

relatively small or without changes in the fatty acid composition of brain PL. Therefore, it appears that the brain is not only more resistant to modification of fatty acid profile by dietary manipulation, but also that changes in ECs and congeners seem to be achievable with extreme changes in the diet and or in physiopathological conditions.

The decrease of AEA in hypothalamus along with the increase of peripheral levels of OEA may have important consequences on body composition [18]. It is therefore attempting to suggest that the decrease of AEA and increase of peripheral OEA may contribute to the decrease of body weight and relative growth retardation.

EFAD is rare in healthy adults and children who consume a varied diet with adequate intake of essential fatty acids. Clinicians should be aware of the risk of EFAD in specific populations that may suffer from malabsorption syndromes, or have other reasons that severely limit fat intake, absorption, or metabolism. A recent concerning trend is the increasing incidence of parenteral nutrition product shortages, including vitamins and minerals, but also lipid injectable emulsion [30]. This has led to new populations at risk for EFAD, and clinicians must be aware of these shortages and the risks posed to patients dependent on parenteral nutrition. Therefore, future studies should aim to evaluate whether in humans changes in ECs and congeners biosynthesis may contribute to EFAD pathological consequences.

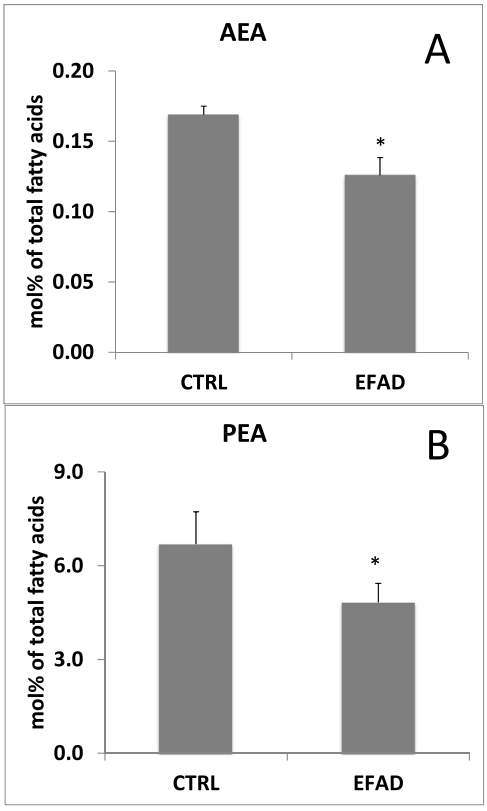

Fig. 4. Anandamide (AEA) (panel A), and palmitoylethanolamide (PEA) (panel B) levels in the hypothalamus of rats fed control diet (CTRL) or essential fatty acid deficient diet (EFAD), values are expressed as mol% of total fatty acids. * Significantly different from control ($p < 0.05$).

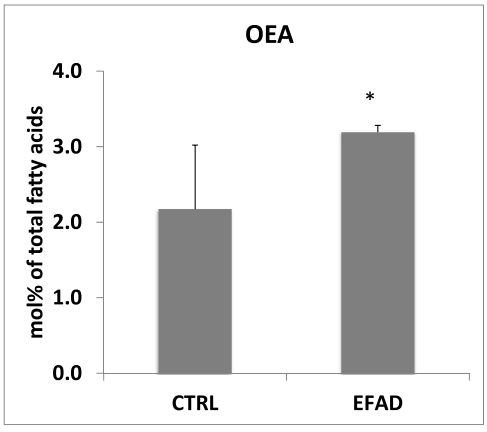

Fig. 5. Oleoylethanolamide (OEA) levels in the adipose tissue of rats fed control diet (CTRL) or essential fatty acid deficient diet (EFAD), values are expressed as mol% of total fatty acids. * Significantly different from control ($p < 0.05$).

Author statement

The authors have no competing interests to declare and that the work described has not been published previously.

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